RESEARCH PLAN PROPOSAL

ROLE OF OXIDATIVE STRESS IN COPPER POISONING AND ITS POSSIBLE REVERSAL BY A POTENT THIOL CHELATOR

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Doctor of Philosophy

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Submitted by

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INTRODUCTION

Review of Literature

Copper is an essential trace mineral that is vitally important for both physical and mental health. Copper has a role in bones and connective tissue, energy production in the cells , immune response, the glandular system , reproductive system, (Angelova, *et al*., 2011) and nervous system (L. Wilson, 2011)

The problem usually occurs when there is too much of it in the soft tissues of the body. Copper imbalances occur when a person becomes copper-toxic, copper-deficient or to have a condition called bio unavailable copper. These conditions occur due to various reasons related to food and nutrition; metabolic type or body chemistry, w.r.t. fast, slow and mixed oxidisors or deficiency of the copper-binding proteins, ceruloplasmin or metallothionein, responsible for copper transport in the body. Without sufficient binding proteins, unbound copper may circulate freely in the body, where it may accumulate primarily in the liver, brain and female organs.

Copper Homeostasis and Pathology of Copper-induced Injury

Copper's essentiality as a unique trace element is due to its ability to act as an electron donor or acceptor as its oxidation state fluxes between Cu^{+1}(cuprous) and Cu^{+2} (cupric). (Ralph and McArdle, 2001). Copper participates in a wide variety of redox reactions in numerous biologic processes. Key examples include copper-dependent enzymes including cytochrome-C oxidase in the mitochondrial electron transport system; Cu/Zn-superoxide dismutase, which is important for free radical detoxification, hephaestin, a ferroxidase that influences intestinal iron absorption; serum ceruloplasmin that functions as a circulating ferroxidase; protein-lysine-6 oxidase, essential for collagen and elastin cross linking; and blood clotting factors V and VIII. (Tapiero *et al.*, 2003)

Copper enters the body through the digestive tract, and under normal condition, is absorbed in the stomach and upper small intestine (Stern, *et. al*, 2007) , concentrated in the liver, and incorporated into cerulosplasmin or excreted into bile (Sass-Kortsak, 1965). A transporter protein on the cells of the small bowel, copper membrane transporter 1 (CMT1), carries copper inside the cells, where some is bound to metallothionein and remaining is carried by ATOX1 to

an organelle known as the trans-Golgi network. Due to elevated concentration of copper, an enzyme called ATP7A releases copper into the portal vein to the liver. Liver cells also carry the CMT1 protein, and metallothionein and ATOX1 bind it inside the cell, but here ATP7B links copper to ceruloplasmin and releases it into the bloodstream, removing excess copper by secreting it into bile.

The oxidation potential of copper is responsible for some of its toxicity. At high concentrations copper is known to produce oxidative damage to biological systems, including peroxidation of lipids or other macromolecules (Bremner, 1998).

Copper accumulates in the hepatocytes in liver tissue; where due to its highly reactive redox capacity induces cellular damage through oxidative stress (Valko *et al.*, 2005). In addition, high intracellular copper levels lead to a conformational change in the anti apoptotic protein, X-linked inhibitor of apoptosis (XIAP). This change increases its degradation and thereby decreases its ability to inhibit caspase-3 leading to a lower apoptotic threshold and cell death (Mufti *et al.*, 2006). When the amount of copper in the liver overwhelms the proteins that normally bind it, it causes oxidative damage through a process known as Fenton chemistry; this damage eventually leads to chronic active hepatitis, fibrosis and cirrhosis. The liver also releases copper into the bloodstream that is not bound to ceruloplasmin. This free copper precipitates throughout the body but particularly in the kidneys, eyes and brain.

In the brain, most copper is deposited in the basal ganglia, particularly in the putamen and globus pallidus (together called the lenticular nucleus); these areas normally participate in the coordination of movement and also play a significant role in neuro cognitive processes such as the processing of stimuli and mood regulation (Lee and Gr, 1999). Damage to these areas produces the neuropsychiatric symptoms. Scientific evidences suggest that high levels of free (non-ceruloplasmin bound) copper have a direct effect on either oxidation of hemoglobin, inhibition of energy-supplying enzymes in the red blood cell, or direct damage to the cell membrane.

The pathogenesis of copper toxicity is outlined in Fig. 2.

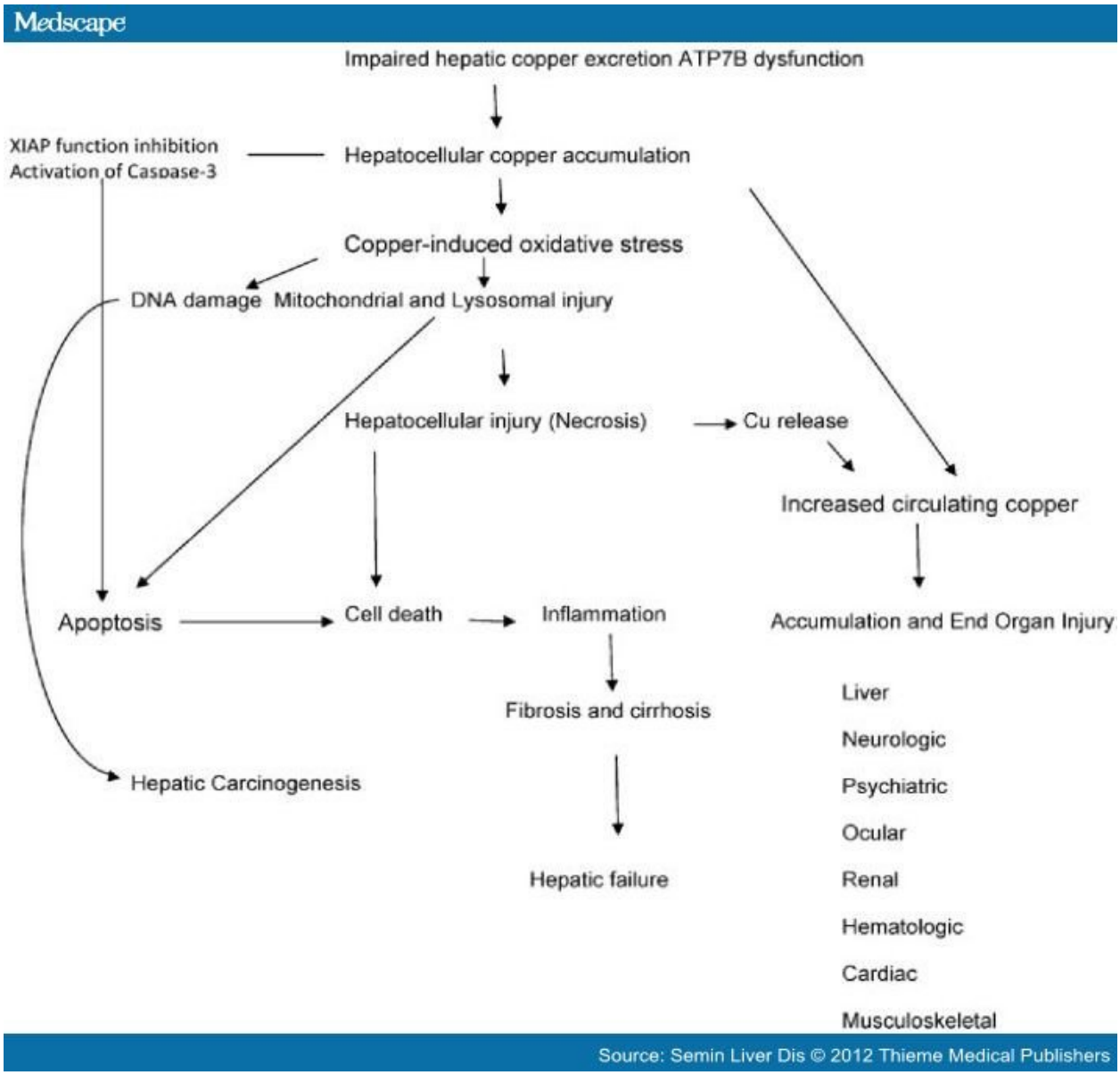


Figure 2: *Pathogenesis of copper toxicity. The main pathways of copper overload/toxicity is through both a direct oxidative stress mechanism with lipid peroxidation of membranes, mitochondria, and DNA; and also from unregulated apoptosis resulting in cell death from copper-induced changes in the anti-apoptotic protein, X-linked inhibitor of apoptosis (XIAP), and its loss of inhibitory control of caspase-3 with copper accumulation.*

[*Source:* Rosencrantz R, Schilsky M. Wilson disease: pathogenesis and clinical considerations in diagnosis and treatment. *Semin Liver Dis.* 2011 Aug;31(3):245-59. Epub 2011 Sep 7]

Treatment therapy of Wilson's disease, to treat copper toxicity, includes the replacement of serum ceruloplasmin and the elimination of the excess tissue copper by the use of a specific chelating agent. Copper chelating agents that increase urinary excretion of copper are the first-line treatment for Wilson disease.

Effective medications include D-penicillamine, trientine, zinc salts, tetrathiomolybdate, and dimercaprol. Chelating agents like **BAL**(2,3 dimercaptrol), **versene** (ethyl- endiamine tetra-acetic acid) and, more recently, penicillamine have been advocated as effective agents (Cumings, 1948; Brown and Porter, 1951).

- **D Penicillamine (Chelator).** 3-Mercapto-D-valine.

It is used as an antirheumatic and as a chelating agent, since 1950s and as a first-line therapy for Wilson disease (Merle *et al.*, 2007)

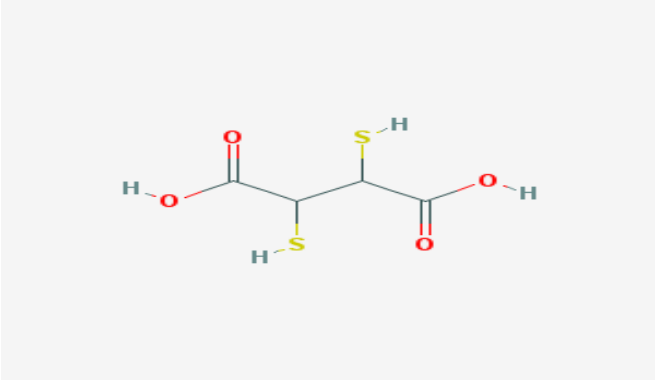
- **Trientine (Chelator)** (Triethylenetetramine; *N*,*N*'-Bis(2-aminoethyl)-1,2- ethanediamine) is the usual second-line treatment for individuals who cannot tolerate penicillamine.
- **Zinc (metallothionein inducer).** High-dose oral zinc interferes with absorption of copper from the gastrointestinal tract by inducing enterocyte metallothionein, which preferentially binds copper from the intestinal contents and is lost in the feces as enterocytes are shed in normal turnover. **Zinc** is now the recommended therapy for long-term management of the disease (Brewer GJ, 2001).
- **Ammonium tetrathiomolybdate,** (NH4)2MoS4, **(chelator) is a therapy under investigation,** which interferes with copper absorption from the intestine and binds plasma copper with high affinity. Copper depletion can occur with this treatment in long term.

Recently a new drug, **MiADMSA** was used against chronic arsenicosis, which can be a possible treatment for Wilson's disease. Its major drawback is the essential metal loss in terms of copper (Flora and Pachauri, 2010). This property can be utilized as a therapeutic strategy against Wilson's disease to remove excessive copper from various target organs, and is one of our main objective in this research.

RATIONALE FOR THE RESEARCH WORK:

DMPS and DMSA are water soluble chemical analogs of dimercaprol (BAL). In contrast to BAL, they have less toxicity, greater water solubility, limited lipid solubility and are effective when given orally (Aposhian, 1983).

Recently some mono and diesters of DMSA especially the higher analogues have been developed and tried against cases of experimental heavy metal poisoning. Mono and dimethyl esters of DMSA that have been studied experimentally with the aim of enhancing tissue uptake of chelating agents. In order to make the compounds more lipophilic, the carbon chain length of the parent DMSA was increased by controlled esterification with the corresponding alcohol (methyl, ethyl, propyl, isopropyl, butyl, isobutyl, pentyl, isopentyl and hexyl.



DMSA: Meso 2,3-Dimercaptosuccinic acid

(Molecular Formula: $C_4H_6O_4S_2$; Molecular Weight: 182.21804)

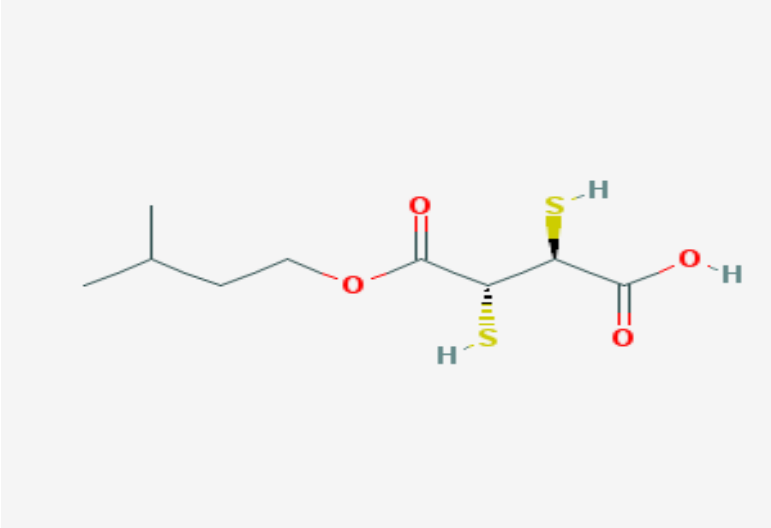
Thiols are known to act as protectants in the biological system for their involvement in a number of metabolic regulations. A new and potent vicinal thiol-chelating agent, monoisoamyl 2,3-dimercaptosuccinic acid (MiADMSA), an analog of meso 2,3-dimercaptosuccinic acid, could act as a prooxidant (because of its lipophilic character) or antioxidant (because of thiol moiety) that could supplement its chelating properties and produce effective clinical recoveries in the treatment of metal intoxication. MiADMSA administration, was found to be safe in rats. (Flora *et al.*, 2007)

MiADMSA could reverse most of the arsenic-induced altered variables to various extents, however, DNA damage remained unaffected (Dwivedi *et al.*, 2011). Similarly, in

Galium-Arsenic poisoning, MiADMSA has shown a promising role. Significant loss of copper after MiADMSA administration, however, was of concern and requires further exploration (Flora *et al.*, 2004). Monoisoamyl ester of DMSA (MiADMSA) has been found to be the most effective (Flora *et al.*, 2002, Flora *et al.*, 2004). Mehta and Flora reported for the first time the comparison of different chelating agents (3 amino and 4 thiol chelators) on their role on metal redistribution, hepatotoxicity and oxidative stress in chelating agents induced metallothionein in rats. MiADMSA also potentate the synthesis of metallothionein in liver and kidneys and GSH levels in liver and brain and also significantly reduced the GSSG levels in tissues.

The property of MiADMSA, to promote loss of copper in arsenic toxicity, can be utilized as a therapeutic strategy to remove excessive copper from various target organs.

Chemical Structure: Monoisoamyl-2,3-dimercaptosuccinate

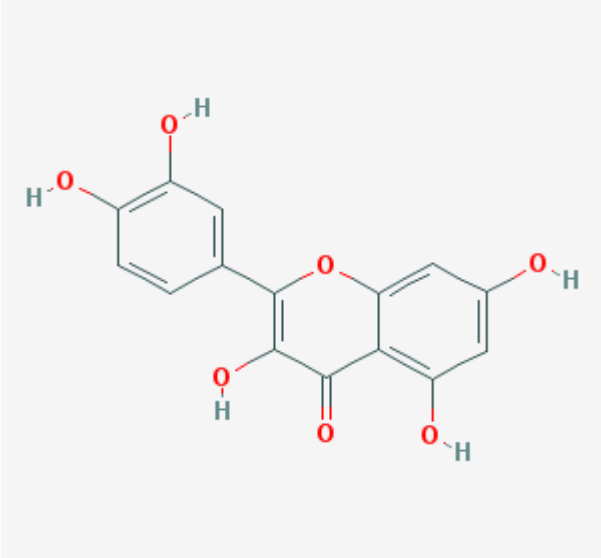


Molecular Formula: $C_9H_{16}O_4S_2$ Molecular Weight: 252.35094

Adjunct therapy with antioxidants, have shown to be beneficial in reducing the damage caused by free radicals (Van den Berghe *et al.*, 2009). Anti oxidants (AOX) are substances which inhibit or delay oxidation of a substrate. Anti oxidant molecules are thought to play a crucial role in counteracting free radical induced damage to macro molecules. Nutritional anti oxidants act through different mechanisms: 1) directly utilize free radicals. 2) reduce the peroxide concentration and repair oxidized membranes and 3) quench iron to decrease ROS production via lipid metabolism, short chain free acids and cholesterol esters neutralize ROS[43].

Flavonoids exert powerful chain-breaking antioxidant activity and vitamin C-sparing action. They possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antiviral, antithrombotic and anticarcinogenic activities. **Quercetin,** a Flavonol, is the glycone form of a number of other flavonoid glycosides, and is one of the most frequently studied dietary flavonoid. It is an excellent free radical scavenging anti oxidant and is a more potent anti oxidant Quercetin and rutin has a positive influence on growth and antioxidant defense system of a human hepatoma cell line (HepG2) (Aloa *et al*.,2006).

Quercetin Chemical Structure



The effect of copper removal in by MiADMSA, a chelator, can be supplemented with the anti oxidative property of Quercetin, a flavonol. The following objective is proposed.

OBJECTIVES OF RESEARCH

1. To study the toxicity of copper particularly the effects on biochemical variables in blood, liver and brain.

2. To study the efficacy of various chelating agents (DMSA and MiADMSA) in improving the biochemical alterations (copper sensitive and copper specific bio markers) following chronic exposure to copper.

3. Effects of co-administration of antioxidants like Querecetin during chelation treatment on copper tissue oxidative stress.

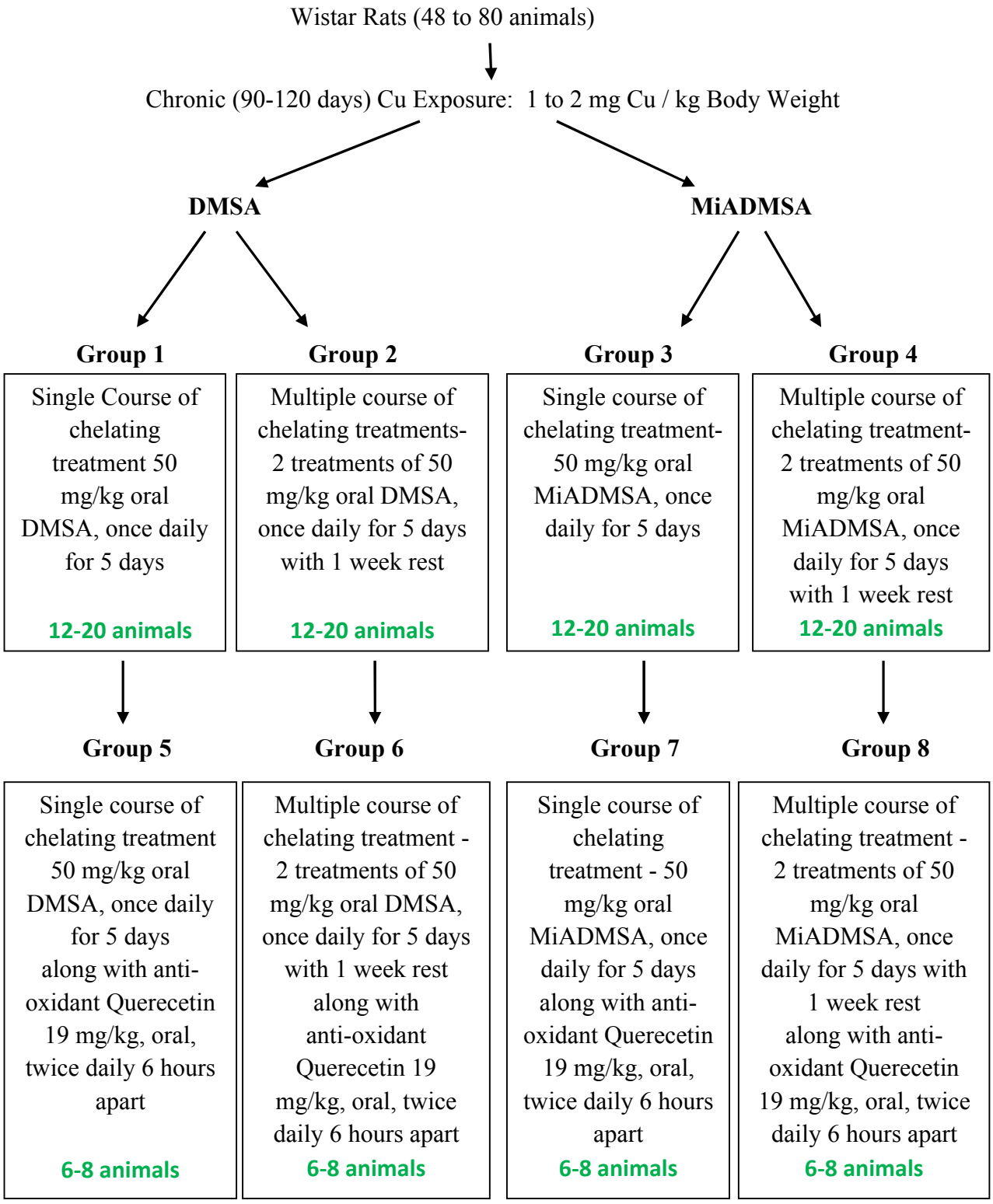
PROPOSED METHODOLOGY OF THE WORK

Animals and treatment:

Male wistar rats of approximate weight 60-70 gm will be used as the animal model. The animals will be exposed chronically to copper as in drinking water (1 or 2 mg/kg) for 3-4 months. Animals pre-exposed to copper will be treated for single or multiple course of 5 days chelation therapy (DMSA, MiADMSA).

Co-administration of some antioxidants like Querecetin (19 mg / kg orally with drinking water) which are known for scavenging properties of free radicals with chelating agents too will be assessed.

PLAN OF WORK


Wistar Rats (48 to 80 animals)
Chronic (90-120 days) Cu Exposure: 1 to 2 mg Cu / kg Body Weight
DMSA
MiADMSA
Group 1
Single Course of chelating treatment 50 mg/kg oral DMSA, once daily for 5 days
12-20 animals
Group 2
Multiple course of chelating treatments- 2 treatments of 50 mg/kg oral DMSA, once daily for 5 days with 1 week rest
12-20 animals
Group 3
Single course of chelating treatment- 50 mg/kg oral MiADMSA, once daily for 5 days
12-20 animals
Group 4
Multiple course of chelating treatment- 2 treatments of 50 mg/kg oral MiADMSA, once daily for 5 days with 1 week rest
12-20 animals
Group 5
Single course of chelating treatment 50 mg/kg oral DMSA, once daily for 5 days along with anti-oxidant Querecetin 19 mg/kg, oral, twice daily 6 hours apart
6-8 animals
Group 6
Multiple course of chelating treatment - 2 treatments of 50 mg/kg oral DMSA, once daily for 5 days with 1 week rest along with anti-oxidant Querecetin 19 mg/kg, oral, twice daily 6 hours apart
6-8 animals
Group 7
Single course of chelating treatment - 50 mg/kg oral MiADMSA, once daily for 5 days along with anti-oxidant Querecetin 19 mg/kg, oral, twice daily 6 hours apart
6-8 animals
Group 8
Multiple course of chelating treatment - 2 treatments of 50 mg/kg oral MiADMSA, once daily for 5 days with 1 week rest along with anti-oxidant Querecetin 19 mg/kg, oral, twice daily 6 hours apart
6-8 animals

Biochemical assays:

After the above treatment, the animals will be sacrificed. Blood, liver, kidneys and brain will be removed for the following biochemical and metal analyses. The methods to be adopted for the estimation of these variables are given in the parenthesis-

Blood – Ceruloplasmin activity in blood/ plasma,
δ-aminolevulinic acid dehydratase (ALAD) (Berlin and Schaller, 1974),
Glutathione (GSH) (Ellman, 1959 modified by Jollow *et al*., 1974),
Reactive Oxygen species (ROS) (Socci *et al.*, 1999),
Superoxide dismutase (SOD) (Kakkar *et al.* ,1983),
RBC and WBC counts,
Haemoglobin and packed cell volume.

Liver – Induction of Metallothioneine, ALAD,
Reduced and oxidised Glutathione (GSH and GSSG) (Hissin and Hilf, 1973),
Thiobarbituric acid reactive substances (TBARS) (Onkawa and Onishi, 1979),
Catalase (Aebi, 1984),
Superoxide dismutase

Brain – Acetylcholine esterase (AChE) (Ellman *et al.*, 1961),
Monoamineoxidase (MAO) (Wurtman and Axelrod, 1969),
Glutathione peroxidase (GPx) (Flohe and Gunzler, 1984),
Glutathione S-transferase (GST) (Habig *et al.*, 1974),

Histoptahological observations: -

Effects of copper exposure on cellular tissues will be studied by Histopathological (light and EM) method

Metal estimation: Liver, kidneys, brain and blood – copper (Parker *et al*.,1967)

What will be achieved from the study?

By using MiADMSA to remove copper toxicity, we will be able to 1) deduce the effectiveness of this chelator on copper removal, 2) see how it affects the biochemical parameters of the main target organs like, blood, liver and brain in terms of removing the oxidative stress caused by copper and 3) deduce if administration of MiADMSA, at this dose, is safe for use.

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